POSSIBLE ROLES OF SUBMANDIBULAR SALIVARY GLANDS ON OVARIAN STEROIDS RESPONSIVENESS OF MOUSE MAMMARY GLANDS

I. S. Yuh[1] and B. B. Jang

Department of Dairy Science, College of Animal Agriculture, Kangwon National University, Chuncheon, Korea 200-701

Summary

This experiment was to determine the effect of sialoadenectomy on the ability of the mammary gland development to response to ovarian steroids, estrogen and progesterone, stimulus *in vivo*. Body weights did not differ between sham-operated and sialoadenectomized mice within 0 to 18 day estradiol + progesterone (E + P) injection ($p > 0.05$). Sialoadenectomy reduced mammary development scores from 4.6 to 3.9 or from 4.4 to 3.8 in comparison with those of sham-operated mice for the 12 or 18 day E + P injection ($p \leq 0.05$), however, sialoadenectomized mice with 0, 1, 3 or 6 day of E + P injection slightly decreased mammary development scores relative to those of sham-operated mice. These results indicate that the endocrine factor secreted from submandibular salivary gland appears to be required for the mammary development to respond fully to estradiol and progesterone. Similar results were obtained in the measurement of mammary DNA contents. Mammary DNA contents of sialoadenectomized mice were significantly decreased relative to those of sham-operated mice for the 6, 12 or 18 day E + P injections. Overall results suggest that salivary gland-secreted endocrine factor, presumably epidermal growth factor (EGF), was mammogenic and should interact with ovarian steroids in mammary development.

(**Key Words** : Sialoadenectomy, EGF, Estrogen, Progesterone, Mammary Development)

Introduction

Epidermal growth factor (EGF) stimulates proliferation of the mammary epithelia in explants, whole mammary gland, and cell cultures (Tonelli and Sorof, 1980; Yang et al., 1980; Taketani and Oka, 1983a,b). The concentration of EGF in both serum and submandibular salivary glands increases during pregnancy in mice (Kurachi and Oka, 1985). Indirect evidence for a stimulatory role of EGF in mammary development was observed by sialoadenectomy, which removed the major source of EGF in normal mouse mammary gland (Okamoto and Oka, 1984; Sheffield and Welsch, 1987). Sheffield (1990) observed that sialoadenectomy, which has previously been shown to depress mammary gland development, alters the endocrine status of mice, such that serum from sialoadenectomized mice is less effective than sham-operated mice in inducing DNA synthesis by mammary epithelial cells. In addition, Sheffield and Yuh (1988) observed that estrogen and progesterone-induced DNA synthesis of bovine mammary tissue transplanted to athymic nude mice was reduced by sialoadenectomy and restored by EGF. Estrogen and progesterone can influence EGF production and secretion. Estrogen and progesterone treatment of animals can increase salivary gland EGF content, level of mammary gland EGF receptors (Vonderhaar, 1984) and increase serum EGF levels (Sheffield and Welsch, 1987).

The objective of this study is to further determine the effect of sialoadenectomy on the ability of the mammary gland development to response to estrogen plus progesterone stimulus *in vivo*.

Materials and Methods

Total 72 female ND/4 mice (4 weeks of age) were used for this experiment at three different times (24 mice on each occasion). All mice were ovariectomized. Half of ovariectomozed mice were sham-operated and the remainder were sialoadenectomized (submandibular

[1]Address reprint requests to Dr. I. S. Yuh, Department of Dairy Science, College of Animal Agriculture, Kangwon National University, Chuncheon, Korea 200-701.

Received February 14, 1996
Accepted July 10, 1996

salivary glands removed, other salivary gland left in place). After a 2 week surgical recovery period, mice were subcutaneousely injected daily for 18, 12, 6, 3 or 0 days with saline (control) or 17β-estradiol (1 μg/day) + progesterone (1 mg/day, E + P, Sigma Chemical Co., St. Louis, MO). The day after the last injection, mice were killed by decapitation and body weights were measured. One inguinal (fourth) mammary gland was removed, fixed, stained with carmine-alum, and mammary development score was rated as described previously on a scale of 1 to 6 using the following criteria (Welsch and Gribler, 1973): 1 = few ducts, few or no end buds; 2 = moderate duct growth, moderate number of end buds; 3 = numerous ducts and branches, many end buds; 4 = numerous ducts and branches, minimum lobule-alveolar development; 5 = numerous ducts and branches, moderate lobule-alveolar growth; and 6 = numerous ducts and branches, dense lobule-alveolar development as in late pregnancy. Lymph nodes were carefully removed from the contralateral fourth mammary glands. The mammary glands were homogenized in saline, and nucleic acids precipitated with 10% trichloroactic acid (TCA). Lipids were removed by sequential washes with methanol : chloroform (2 : 1, vol : vol), ethanol and ethyl ether. Deoxyribonucleic acid (DNA) was extracted by heating with 5% perchloric acid (70℃, 30 min). An aliquot of the extract was neutralized and used to determine DNA by the diphenylamine reaction (Burton, 1956).

Statistical analysis

Data were analyzed by analysis of variance. Planned comparisons were used to compare sialoadenectomy with sham-operation at the given E + P injection days or to compare effects of E + P injection days for sialoadenetomy or sham-operation. Unless otherwise noted, significance was set at $p < 0.05$. In all cases, two-sided tests were performed (Gill, 1978).

Results

Body weights of sham-operated mice were 23.8, 26.1, 26.1, 25.4, 24.0 and 24.4 g (Pooled SE = 1.19 g) for 0, 1, 3, 6, 12 and 18 day E + P-injected mice, respectively. In sialoadenectomized mice, body weights were 24.4, 24.6, 24.8, 24.1, 24.7 and 24.3 g for 0, 1, 3, 6, 12 and 18 day E + P injected mice, respectively (figure 1). Body weights did not differ between sham-operated and sialoadenectomized mice within the indicated E + P injection day ($p > 0.05$). Body weights also did not differ among E + P injection days within sham-operated or sialoadenectomized mice. Sialoadenectomy reduced mammary development score from 3.6 to 3.1, from 4.6 to 3.9 or from 4.4 to 3.8 in comparison with those of sham-operated mice for the 6, 12 or 18 day E + P injection, respectively (Pooled SE = 0.20, figure 2). In addition, 3, 6, 12 or 18 day E + P injection increased mammary development scores relative to those of 0 day E + P injection in both of sham-operated and sialoadenectomized mice. In figure 3, the representative whole-mount examination of mammary glands showed that sialoadenectomy reduced mammary gland development. Similar results were observed in the measurement of mammary DNA contents (figure 4). In sialoadenectomized mice, mammary DNA contents were decreased from 280.2 μg/gland to 248.5 μg/gland, 314.5 μg/gland to 258.0 μg/gland or 309.3 μg/gland to 264.9 μg/gland (Pooled SE = 14.3 μg/gland) in comparison with those of sham-operated mice for the 6, 12 or 18 day E + P injection, respectively. Injection of E + P for 6, 12 or 18 days increased DNA content of sham-operated mice from 175.2 μg/ gland in 0 day E + P injection to 280.2, 314.5 or 309.3 μg/gland, respectively ($p \leq 0.05$). In sialoadenectomized mice, 6, 12 or 18 day E + P injection also increased mammary DNA contents from 165.2 μg/gland

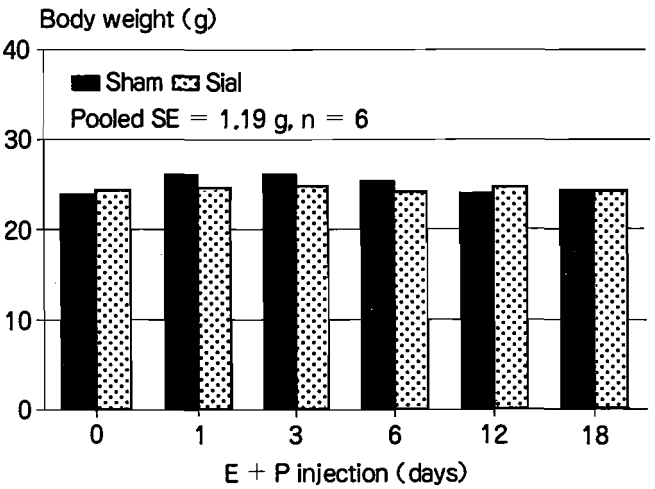

Figure 1. Body weights of mice that were sham-operated (Sham) or sialoadenectomized (Sial). There were no statistical differences between sham-operated and sialoadenectomized mice for the given estrogen + progesterone (E + P) injection days ($p > 0.05$). In addition, body weights were not different among E + P injection days within sham-operated or sialoadenectomized mice ($p > 0.05$, number of mice (n) = 6).

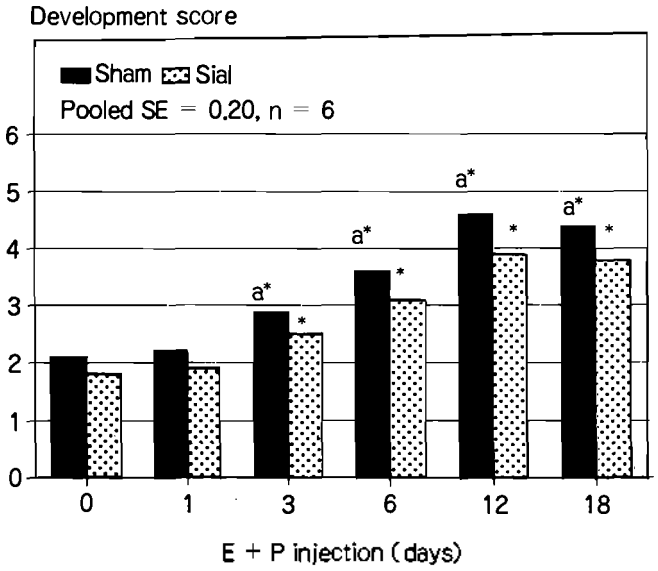

Figure 2. Mammary gland (No. 4, inguinal) whole mount development scores from mice that were sham-operated (Sham) or sialoadenectomized (Sial). Statistical comparisons: * = E + P injection days increased development score relative to 0 day of E + P injection in sham or sialoadenectomized mice ($p \leq 0.05$): a = sialoadenectomy reduced mammary development score relative to sham operation for the given E + P injection days ($p \leq 0.05$, Pooled SE = 0.20, number of mice (n) = 6).

in 0 day E + P injection to 248.5, 258.0 or 264.9 μg/gland, respectively ($p \leq 0.05$).

[B]

[C]

[A]

[D]

Figure 3. Representative whole mounts of mouse mammary glands. [A] sham operated and saline treated for 18 days: [B] sialoadenectomized and saline treated for 18 days: [C] sham operated, 6 day estrogen + progesterone treated: [D] sialoadenectomized, 6 day estrogen + progesterone treated (X15).

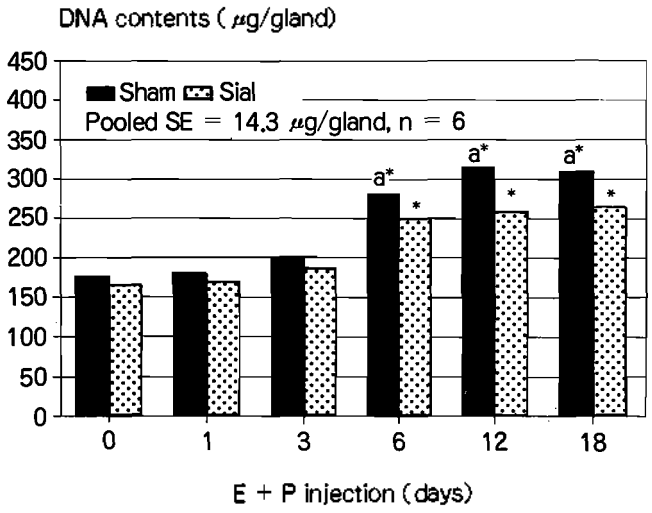

Figure 4. Mammary DNA of mice that were sham-operated (Sham) or sialoadenectomized (Sial). Statistical comparisons: * = E + P injection days increased mammary DNA relative to sham or sialoadenectomy control (0 day E + P injection, $p \leq 0.05$); a = sialoadenectomy reduced mammary DNA relative to sham-operation for the given E + P injection days ($p \leq 0.05$, Pooled SE = 14.5 μg /gland, number of mice (n) = 6).

Discussion

17β-estradiol was required *in vivo* for both ductal growth during sexual maturation and lobuloalveolar development during pregnancy (Topper and Freeman, 1980). In concert with progesterone, estrogen controlled successive rounds of mammary cell proliferation and lobuloalveolar development from glands removed from estrogen- and progesterone-primed mice (Tonelli and Sorof, 1980). Results of this study also demonstrated that 6, 12, or 18 day E + P injections increased mammary development score or mammary DNA contents in sham-operated and sialoadenectomized mice. Ovarian steroids appeared to be mitogenic and were necessary for normal mammary growth with or without salivary glands. Without ovarian steroids, sham-operated mice tended to increase mammary development score and DNA contents relative to those of sialoadenectomized mice, however, no statistical difference was shown ($p > 0.05$). In this result, we could not rule out the possibility of incomplete removal of salivary gland during the operation causing low response in mammary development or the possibility of minor source of endocrine factor such as EGF in submandibular salivary gland rather than other tissues. In figure 1, sialoadenectomy did not change body weight in comparison with that of sham-operated mice for the experimental period. This results suggest that the effect of sialoadenectomy in mammary growth was probably due to endocrine factor rather than nutritional factors. In addition, sham-operated mice significantly increased mammary development score and DNA contents relative to those of sialoadenectomized mice from 6 day to 18 day E + P injection ($p \leq 0.5$), indicating that the endocrine factor secreted from submandibular salivary gland appear to be required for the mammary development to respond fully to estradiol and progesterone. Serum concentration of EGF in bovine mammary tissue transplanted to athymic nude mice was significantly reduced by sialoadenectomy but was detectable in sham-operated mice (Sheffield and Yuh, 1988). The importance of salivary gland secreting presumably EGF, was also suggested by Sheffield (1990) that serum from sialoadenectomized mice is less effective than that of sham-operated mice in inducing DNA synthesis by mammary epithelial cells. In particular, this salivary gland-secreted endocrine factor, EGF, should interact with estrogen and progesterone response in mammary development. Several studies indicated that ovarian steroids (estrogen and progesterone) increased tyrosine phosphorylating activity (Sheffield et al., 1987) and increased mammary EGF-receptor content (Sheffield, 1988; Vanderboom and Sheffield, 1993) suggesting that the mammary EGF receptor regulated, at least in part, by ovarian steroids.

Literature Cited

Burton, K. A. 1956. Study of the conditions and mechanisms of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. Biochem. J. 62:315-323.

Gill, J. L. 1978. Design and Analysis of Experiments in the Animal and Medical Sciences. Ames. IA, Iowa State University Press, Vol. 1, 1978.

Kurachi, H. and T. Oka. 1985. Changes in epidermal growth factor concentration of submandibular gland, plasma and urine of normal and sialoadenectomized female mice during various reproductive stages. J. Endocrinol. 106:197-202.

Okamoto, S. and T. Oka. 1984. Evidence for physiological function of epidermal growth factor: Progestational sialoadenectomy of mice decreases milk production and increases offspring mortality during lactation period. Proc. Natl. Acad. Sci. USA. 81:6059-

6063.

Sheffield, L. G. 1990. Effect of sialoadenectomy on the ability of mouse serum to induce deoxyribonucleic acid synthesis in mammary epithelial cells : Possible role of epidermal growth factor. J. Dairy Sci. 73:2087-2092.

Sheffield, L. G. 1988. Estradiol and progesterone modulation of mammary epidermal growth factor receptor. J. Dairy Sci., 71: (suppl. 1.):229 (Abstr.).

Sheffield, L. G., C. F. Aylsworth, C. W. Welsch. 1987. Cyclic nucleotides and protein phosphorylation in mouse mammary glands: Effects of estrogen and progesterone administration *in vivo*. Proc. Soc. Exp. Biol. Med. 185:283-290.

Sheffield, L. G. and C. W. Welsch. 1987. Influence of submandibular salivary glands on hormone responsiveness of mouse mammary glands. Proc. Soc. Exp. Biol. Med. 186:368-377.

Sheffield, L. G. and I. S. Yuh. 1988. Influence of epidermal growth factor on growth of bovine mammary tissue in athymic nude mice. Domestic Animal Endocrinol. 5:141-147.

Taketani, Y. and T. Oka. 1983a. Epidermal growth factor stimulates cell proliferation and inhibits functional differentiation of mouse mammary epithelial cells in culture. Endocrinology 113:871-877.

Taketani, Y. and T. Oka. 1983b. Biological action of epidermal growth factor and its functional receptors in normal mammary epithelial cells. Proc. Natl. Acad. Sci. USA 80:2647-2650.

Tonelli, Q. J. and C. S. Sorof. 1980. Epidermal growth factor requirement for development of cultured mammary gland. Nature 285:250-252.

Topper, T. J. and C. S. Freeman. 1980. Multiple hormone interactions in the developmental biology of the mammary gland. Physiol. Rev. 60:1049-1106.

Vanderboom, R. J. and L. G. Sheffield. 1993. Estrogen enhances epidermal growth factor-induced DNA synthesis in mammary epithelial cells. J. Cellular Physiology 156:367-372.

Vonderhaar, B. K. 1984. Hormone and growth factors in mammary gland development. In: Veneziale, C. M. (ed) Control of cell growth and proliferation. Van Nostrand-Reinhold, Princeton, NJ, p. 11.

Welsch, C. W. and C. Gribler. 1973. Prophylaxis of spontaneously developing mammary carcinoma in C3H/HeJ female mice by suppression of prolactin. Cancer Res. 33:2939-2946.

Yang, J., R. Guzmanm, J. Richards, N. Imagawa, K. McCormick, and S. Nandi. 1980. Growth factor and cyclic nucleotide-induced proliferation of normal and malignant mammary epithelial cells in primary culture. Endocrinology 107:35-41.